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Uterine Environment and Breed Effects on Erythropoiesis and Liver Protein Secretion in Late Embryonic and Early Fetal Swine[1]

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ABSTRACT

In this study we investigated erythropoiesis and fetal liver protein secretion during late embryonic (Day 24 and Day 30) and early fetal (Day 40) development in pigs from domestic white crossbred (WC) gilts with a normal (intact; INT) or crowded (unilateral hysterectomized/ovariectomized; UHO) uterine environment, or from prolific Chinese Meishan (MS) gilts. Increased fetal weight, fetal liver weight, placental weight, total red blood cells, hematocrit, blood hemoglobin content, and maternal plasma erythropoietin (EPO) levels were observed as gestation advanced. Cultured fetal liver secretion of transferrin and a protein of M_r 12 500 and pI 7.5 also increased as gestation advanced. Fetal plasma EPO declined between Day 30 and Day 40. Differential counts of circulating erythroid precursors revealed a decline in basophilic erythroblasts and polychromatic erythroblasts between Day 24 and Day 40, an increase in orthochromatic erythroblasts on Day 30 followed by a drop on Day 40, and an increase in the percentage of reticulocytes/erythrocytes from < 1.0% to approximately 90% of circulating red blood cells between Day 24 and Day 40. Differences among the treatment groups included a lower fetal survival percentage in UHO (vs. INT or MS) on Day 40, and higher maternal hematocrits, fetal weights, fetal hematocrits, fetal EPO levels, and liver transferrin secretion in WC vs. MS pigs. MS pigs had a lower percentage of polychromatic erythroblasts overall and a higher percentage of orthochromatic erythroblasts on Day 24 followed by a higher percentage of erythrocytes on Day 40 than WC pigs, suggesting a more mature erythron (circulating red blood cells plus erythropoietic tissue) in the MS pigs. Covariate analysis indicated that MS had larger placentae per unit of body weight than did WC. Conclusions were that 1) Days 24–40 of gestation is a critical time for fetal erythropoiesis in pigs as well as survival in a crowded uterine environment, 2) the MS breed may differ in the development of the fetal erythropoietic system because of altered fetal or uterine physiology, and 3) the UHO procedure did not significantly affect erythropoiesis in the fetuses studied but did alter fetal survival and the relationship between fetal weight and both hematocrit and hemoglobin on Day 40.

INTRODUCTION

Embryonic erythropoiesis in vertebrates is characterized by development of pluripotent hematopoietic stem cells from embryonic mesoderm and formation of blood islands in the yolk sac [1, 2]. The production of nucleated erythroid precursors in the yolk sac (primitive erythropoiesis) continues until "seeding" of the newly forming liver by circulating erythroid cells (around Day 10 of gestation in mice, Week 6 in humans). Thereafter, the liver is the major site for definitive erythropoiesis (production of mature non-nucleated red blood cells) until the spleen and bone marrow become major hematopoietic sites in neonatal and postnatal animals [2]. The transition from primitive to definitive erythropoiesis occurs on approximately Day 20 of gestation in swine after the formation of the hepatic anlage on Day 18 [3].

Litter size in swine is controlled by several factors, including ovulation rate, uterine capacity, and fetal survivability [4]. Despite years of research into these mechanisms, increase in litter size in the domestic pig has been slow (0.037 pigs/litter/yr from 1966–1996) [5, 6]. Previous studies attempting to increase litter size in pigs by superovulation [7, 8], genetic selection for ovulation rate [9, 10], or embryo transfer [11, 12] have resulted in dramatically increased fetal numbers by Day 30 of gestation, but litter size at term was increased by only approximately 1.0 pig because of fetal loss later in gestation. These studies and others employing experimental models for uterine crowding indicate that "extra" fetuses are lost beginning between Day 25 and Day 50 of gestation [13–15]. Hallmarks of fetal development in swine at this time include organogenesis [16] and sexual differentiation [16, 17]. Failure of sexual differentiation is unlikely to be lethal, and few organ systems other than circulatory and excretory systems are likely to be functioning on Day 25 of gestation. However, fetal erythropoiesis is being initiated at this time in the liver [3].

Recent studies in mice indicate a requirement of erythropoietic factors for embryonic/fetal survival [18]. Genetic "knockouts" of erythroid transcription factors, erythropoietin (EPO) or EPO receptor block erythroid development and result in severe anemia and embryonic lethality between Day 9.5 and Day 15.5 of gestation [19–28]. Iron, folate, and vitamin B_{12} are also required for erythropoiesis [29] and must come from a maternal source. Because initiation of fetal liver erythropoiesis is occurring during this critical time for fetal survival in swine, failure of erythropoiesis may be involved in fetal mortality and reduction of litter size. However, embryonic/fetal erythropoiesis has not been compared in normal and crowded intrauterine environments or in European and prolific Chinese Meishan (MS) breeds. The MS breed averages 30–40% (4.0) more pigs per litter than do their domestic/occidental counterparts [30], presumably because of increased fetal survivability and ovulation rate [31], and have been used extensively in research for their enhanced reproductive characteristics [4, 32–38]. Increased litter size in MS pigs suggests that the physiology of pregnancy has been altered to alleviate some fetal loss due to intrauterine crowding. Thus, this breed provides a useful experimental model for comparison with occidental breeds when studying suspected mechanisms of fetal loss caused by decreased uterine capacity. The unilateral hysterectomized/ovariectomized (UHO) pig

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also provides a model of intrauterine crowding that can be used for similar purposes. The objectives of the current study were to characterize late embryonic and early fetal erythropoiesis in the pig and investigate the effects of uterine environment and breed.

MATERIALS AND METHODS

Animals and Tissue Collection

Investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by the Society for the Study of Reproduction.

White crossbred (WC) gilts (1/4 Yorkshire, 1/4 Large White, 1/4 Chester White, and 1/4 Landrace) were unilaterally hysterectomized and ovariectomized at approximately 160 days of age using previously described procedures [13]. Twenty WC UHO, 17 WC normal (intact: INT), and 23 MS gilts were observed for behavioral estrus once daily. After at least one estrous cycle of normal length, WC (INT and UHO) gilts (age 262–320 days) were bred. Because of earlier onset of puberty [39] and increases in ovulation rate up to the sixth estrous cycle [4], MS gilts (age 272–308 days) were not bred until after their seventh estrous cycle. Pregnant gilts of each treatment group (INT, UHO, and MS) were slaughtered on either Day 24, 30, or 40 of pregnancy. At slaughter, a maternal blood sample (mixed arterial and venous; approximately 20 ml/gilt) was collected in a 50-ml polypropylene tube containing 1 ml (600 U) heparinized saline. The reproductive tract was removed, the entire length of the uterus was opened along the antimesometrial surface, and blood samples were collected from the umbilical vessels (mixed arterial and venous blood) of each embryo/fetus (for simplicity, embryos and fetuses are hereafter referred to as "fetus," with "fetal" used as a descriptive term) in heparinized syringes. Fetuses were then removed and weighed, and liver tissue from one fetus/gilt was collected for explant culture. Placentae were then removed and weighed, and corpora lutea were counted. Blood smears were made from 2–3 individual fetal blood samples per gilt, and remaining blood samples from Day 24 or Day 30 fetuses from the same gilt were pooled for subsequent analysis because of low volume of blood collected. Fetal survival percentages were calculated by dividing the number of live fetuses (presence of heartbeat, robust appearance) by the number of corpora lutea × 100.

Hematological Measures—Fetal and Maternal

Blood samples were diluted in sterile heparinized saline, and total red blood cells (RBC) were counted using a hemocytometer [40]. To determine hematocrit percentage, small volumes of blood from Days 24 and 30 pooled samples and individual Day 40 and maternal samples were collected in heparinized microhematocrit capillary tubes (Scientific Products, McGaw, IL), centrifuged for 3 min in a microhematocrit centrifuge (Damon IED MB, Needham Heights, MA), and evaluated with a Lancer Spiracrit microhematocrit capillary tube reader (Brunswick Co., St. Louis, MO). Hemoglobin concentration was measured on pooled and individual blood samples in duplicate using a total hemoglobin kit (Sigma, St. Louis, MO). Plots of expected vs. measured concentrations had slopes for maternal ($b = 0.95$) and fetal blood ($b = 0.947$) that did not differ from a value of one. Relative EPO concentrations in fetal and maternal plasma were measured with a heterologous RIA procedure (Diagnostics Systems Laboratories, Webster, TX) using a rabbit antiserum to human EPO. Duplicate 50-μl plasma samples were assayed in a final incubation volume of 100 μl before addition of 0.5 ml of secondary antibody solution. EPO was measured in a single assay with an average intraassay coefficient of variation (CV) of 1.6%. The detection limit of the assay was 0.47 mU/tube. Serial dilutions of fetal and maternal plasma were parallel to the standard curve (data not shown). Accuracy of recovery for five known amounts (0.125–2.5 mU) of EPO added to maternal plasma was 93.6% (data not shown). Cross-reactivity with porcine EPO was not determined by the antibody supplier. However, the antibody does cross-react with rat (87%), sheep (45%), and mouse (39%) EPO. The antibody does not cross-react significantly with other hormones (e.g., human prolactin and insulin, rat FSH, hCG, or epidermal growth factor).

Differential Cell Counts of Erythroid Precursors in Fetal Blood

Smears were prepared from approximately 50 μl of blood samples from two fetuses per gilt, fixed with 100% methanol, and stained with Modified Wright-Giemsa stain (Sigma) for evaluation of erythrocyte and erythroid precursor numbers. Slides were evaluated by two investigators who were unaware of the day or treatment group represented by each slide. At least 100 cells/slide were counted under oil immersion (magnification, ×1000) and classified [28, 40] as either basophilic erythroblast (BE), polychromatic erythroblast (PE), orthochromatic erythroblast (OE), or erythrocyte. In addition, one smear from pooled Day 30 fetal blood samples for each gilt and two smears from individual Day 40 fetal blood samples for each gilt were stained with Accustain (Sigma) to differentiate reticulocytes and erythrocytes. Numbers of cells in the various classifications were converted to a percentage of the total, and the individual percentages of reticulocytes and erythrocytes were estimated from results of the reticulocyte stain evaluation. Results from the two observers were then averaged for statistical analysis. Because of a paucity of erythrocytes in Day 24 smears, no reticulocyte staining was done for that age. Thus, statistical analysis of reticulocytes included only Day 30 and Day 40. Estimates (by correlation analysis) of repeatability between observers were 0.38 for BE, 0.98 for PE, 0.96 for OE, 0.91 for reticulocytes, and 0.98 for erythrocytes.

Two-Dimensional (2D) PAGE Analysis of Fetal Liver Protein Secretion

The liver of one fetus from each gilt was collected into ice-cold minimal essential medium (MEM) with 0.1 times the normal amount of leucine among other modifications [41]. Up to 200 mg of liver tissue was cultured in 5.0 ml of low-leucine MEM with 50 μCi [^{3}H]leucine or 24 h at 37°C under an atmosphere of 50% N_2:45% O_2:5% CO_2. Medium was separated from tissue by centrifugation (1000 × g for 10 min) and then frozen at −20°C until processed further. Conditioned medium from embryonic and fetal livers was dialyzed against 10 mM Tris, 0.02% sodium azide, pH 8.2 (3 changes of 8 L, each overnight) at 4°C. Incorporation of [^{3}H]leucine into nondialyzable macromolecules was determined by scintillation counting of 100 μl of dialysate. A volume of dialysate equal to 10 mg of cultured liver was lyophilized and subjected to 2D-PAGE, Coomassie blue staining, and fluorography for examination of

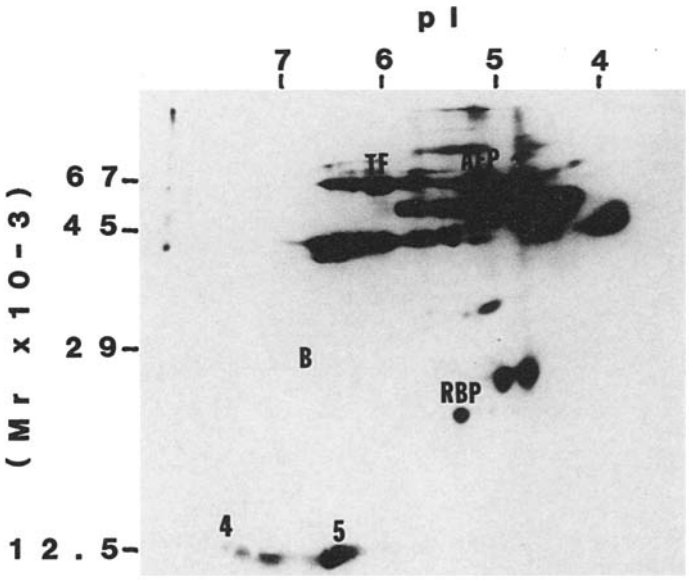

FIG. 1. Representative fluorograph from 2D-PAGE analysis of cultured Day 30 fetal swine liver. Spots punched for scintillation analysis are indicated by labels placed directly above the spot punched from each gel. Spots labeled 4 and 5 are proteins 4 and 5, respectively; B, background spot (no Coomassie staining).

proteins synthesized by the tissue in culture. Quantification of nondialyzable radioactivity contained in individual protein spots was used to determine differences between ages and treatment groups. Fluorographs (Fig. 1) were aligned with the corresponding dried gels, and spots corresponding to transferrin (TF; 80/6 [$M_r \times 10^{-3}$/pI]), alpha-fetoprotein (AFP; 80/5.2), retinol-binding protein (RBP; 20/5.5), two unidentified proteins (4 [12.5/7.5] and 5 [12.5/6.5]), and background (B; no staining) were punched from the gels using a sharpened 7-mm inner-diameter cork borer. Gel punches were solubilized by incubation in 500 μl 30% H_2O_2 at 80°C for 48–72 h, quenched with 500 μl 50 mM ascorbic acid; then 10 ml scintillation fluid was added, and counts per minute (cpm) were determined by scintillation counting. Because nondialyzable radioactivity was not linear with weight of tissue cultured (data not shown), counts from individual spots were expressed as (cpm − cpm B)/total cpm lyophilized.

Statistical Analysis

Data were analyzed using the General Linear Models (GLM) least squares analysis of variance procedures of Statistical Analysis Systems (SAS) [42]. All parameters were averaged within gilt and analyzed in a statistical model that included treatment group, day of gestation, and their interaction. Data for fetal weight, fetal liver weight, placental weight, fetal total RBC count, fetal EPO levels, BE, OE, and all 2D-PAGE data were log-transformed, and data from fetal hemoglobin levels were square root-transformed before analysis to alleviate heterogeneity of variance. When significant effects of day or treatment were detected, data were further analyzed by orthogonal or nonorthogonal contrasts where appropriate. For effects of day, contrasts were 1) Day 24 vs. Day 30, 2) Day 30 vs. Day 40, and 3) Day 24 vs. Day 40. For effects of treatment, contrasts were 1) INT vs. UHO, 2) WC (INT and UHO) vs. MS (if contrast 1 was not significant), and 3) INT vs. MS (if contrast 1 was significant). When an interaction between day and treatment was detected, the treatment contrasts as above were conducted within each day of gestation. For fetal survival percentage, the day-by-treatment interaction was partitioned into four one-degree-of-freedom contrasts using orthogonal comparisons. Analysis of covariance was conducted on fetal, fetal liver, and placental weight data, with fetal weight and placental weight used separately as covariates for the other two parameters and together for fetal liver weight. Regression analysis was conducted on data from Day 40 fetuses to determine relationships between fetal weight and either total RBC counts, hematocrit, hemoglobin, or EPO.

RESULTS

Fetal and Corpora Lutea Numbers and Fetal Survival Percentages

In the interest of brevity, only results with a significant relationship are presented in the *Results* section. Fetal number was higher ($p < 0.01$) in INT vs. UHO but did not differ between INT and MS ($p > 0.05$; Table 1). Corpus luteum numbers were higher ($p < 0.01$) on Day 24 and Day 30 than on Day 40, and they were higher ($p < 0.01$) for MS gilts than for WC gilts (Table 1). No overall effect of day, treatment, or their interaction was observed for fetal survival percentage (Table 1). However, single degree-of-freedom contrasts indicated that an interaction ($p < 0.05$) was present between intact uterine environments (WC INT and MS combined) vs. UHO, and Day 24 and Day 30 combined vs. Day 40. This interaction was due to decreased fetal survival on Day 40 in UHO gilts.

TABLE 1. Fetal numbers, corpora lutea (CL) numbers, and fetal survival percentage.[a]

Parameters	Day of gestation	WC INT	WC UHO	MS
No. of fetuses[b]	24	10.8 ± 1.1 (5)	11.3 ± 1.0 (6)	13.3 ± 0.9 (7)
	30	12.5 ± 1.0 (6)	10.0 ± 0.9 (7)	13.5 ± 0.9 (8)
	40	11.5 ± 1.0 (6)	7.4 ± 0.9 (7)	12.1 ± 0.9 (8)
No. of CL[c]	24	13.2 ± 1.1 (5)	13.3 ± 1.0 (6)	16.4 ± 0.9 (7)
	30	15.0 ± 1.0 (6)	13.3 ± 0.9 (7)	15.8 ± 0.9 (8)
	40	13.2 ± 1.0 (6)	11.0 ± 0.9 (7)	13.9 ± 0.9 (8)
Survival (%)[d]	24	84.7 ± 5.5 (5)	85.2 ± 5.1 (6)	80.4 ± 4.7 (7)
	30	83.0 ± 5.1 (6)	76.2 ± 4.7 (7)	85.4 ± 4.4 (8)
	40	87.8 ± 5.1 (6)	69.9 ± 4.7 (7)	87.9 ± 4.4 (8)

[a] Least-squares means ± SEM; number of pregnant gilts in parentheses.
[b] Fetal number was greater ($p < 0.01$) in INT vs. UHO.
[c] CL numbers were greater ($p < 0.01$) on Day 24 or Day 30 than on Day 40 and were greater ($p < 0.01$) for MS gilts than for WC gilts.
[d] Single degree of freedom interaction contrasts indicated that an interaction was present ($p < 0.05$) between WC INT and MS combined vs. UHO and on Day 24 and Day 30 combined vs. Day 40.

TABLE 2. Fetal, fetal liver and placental weights.[a]

Parameter	Day of gestation	WC INT	WC UHO	MS
Fetal wt. (g)[b]	24	0.43 ± 0.33 (5)	0.41 ± 0.30 (6)	0.38 ± 0.28 (7)
	30	1.45 ± 0.30 (6)	1.46 ± 0.28 (7)	1.29 ± 0.26 (8)
	40	9.79 ± 0.30 (6)	9.20 ± 0.28 (7)	7.96 ± 0.26 (8)
Fetal liver wt. (g)[c]	24	0.02 ± 0.04 (5)	0.02 ± 0.04 (6)	0.02 ± 0.04 (7)
	30	0.17 ± 0.04 (6)	0.17 ± 0.04 (7)	0.16 ± 0.03 (8)
	40	1.06 ± 0.04 (6)	0.90 ± 0.04 (7)	0.91 ± 0.03 (8)
Placental wt. (g)[d]	24	5.5 ± 3.1 (5)	4.3 ± 2.8 (6)	5.9 ± 2.6 (7)
	30	21.9 ± 2.8 (6)	19.6 ± 2.6 (7)	16.4 ± 2.4 (8)
	40	51.2 ± 2.8 (6)	42.3 ± 2.6 (7)	49.0 ± 2.4 (8)

[a] Least-squares means ± SEM; number of pregnant gilts in parentheses.
[b] Fetal weights increased ($p < 0.01$) with day of gestation and were greater ($p < 0.01$) in WC than in MS; fetal weights were greater ($p < 0.01$) in WC vs. MS with placental weight as covariate.
[c] Fetal liver weights increased ($p < 0.01$) with day of gestation; fetal liver weight was greater ($p < 0.05$) in WC vs. MS on Day 24 with placental weight as covariate.
[d] Placental weights increased ($p < 0.01$) with day of gestation; placental weight was greater ($p < 0.01$) in MS vs. WC with fetal weight as a covariate.

Fetal, Fetal Liver, and Placental Weights

Fetal, fetal liver, and placental weights all increased ($p < 0.01$) with day of gestation (Table 2). Fetal weights were higher ($p < 0.01$) in WC than MS and remained higher ($p < 0.01$) when placental weight was the covariate. Placental weights did not differ according to treatment when ANOVA was used without covariate analysis, but they were higher ($p < 0.01$) in MS than in WC when fetal weight was used as a covariate. Fetal liver weights did not differ according to treatment. However, with placental weight as the covariate, fetal liver weight was higher ($p < 0.05$) in WC than MS on Day 24. This difference disappeared when both placental and fetal weights were used as covariates.

Hematological Measures: Fetal

Fetal RBC increased ($p < 0.01$) on both Day 30 and Day 40 (Table 3) and did not differ according to treatment. Fetal hematocrit percentage increased ($p < 0.01$) on both Day 30 and Day 40 and was higher ($p < 0.01$) in WC than in MS (Table 3). Fetal hemoglobin concentrations increased ($p < 0.01$) on Day 30 and Day 40 (Table 3) and did not differ with different treatments. EPO levels were higher ($p < 0.01$) on Day 30 than on Day 40 and were higher ($p < 0.01$) in WC than in MS.

Regression analysis revealed no significant relationship between fetal weight and hematocrit or fetal weight and hemoglobin on Day 40 in either WC INT or MS (intact uterine environment groups), which did not differ. A positive ($p < 0.05$) linear relationship was obtained between these traits in UHO, which differed ($p < 0.05$) from the INT and MS groups combined.

Hematological Measures: Maternal

Total maternal RBC were higher on Day 40 in INT than in UHO ($p < 0.05$) or MS ($p < 0.01$; Table 4). Maternal hematocrit percentage was higher in INT than in UHO ($p < 0.05$) on Day 24 and in WC than in MS on both Day 30 ($p < 0.01$) and Day 40 ($p < 0.01$; Table 4). Maternal hemoglobin concentrations were higher ($p < 0.01$) on Day 24 in INT than in UHO or MS (Table 4). Maternal plasma EPO concentrations were higher ($p < 0.01$) on Day 40 than on Day 30 (Table 4) and did not differ for treatments.

Differential Cell Counts of Erythroid Precursors in Fetal Blood

The percentages of BE and PE decreased ($p < 0.01$) with day of gestation, and PE was higher ($p < 0.01$) in WC than in MS (Table 5). Percentage of OE was higher ($p < 0.01$) on Day 30 than on Day 24 or Day 40 and was higher ($p < 0.01$) in MS or UHO than in INT on Day 24 (Table 5); it did not differ for treatments. Erythrocyte percentage increased ($p < 0.05$) with day of gestation and was higher ($p < 0.01$) on Day 40 in MS than in WC (Table 5). Protein 4, AFP, and RBP were unaffected by day or treatment.

TABLE 3. Hematological measures: fetal.[a]

Parameter	Day of gestation	WC INT	WC UHO	MS
RBC/ul ($\times 10^6$)[b]	24	0.28 ± 0.19 (5)	0.24 ± 0.20 (4)	0.25 ± 0.16 (6)
	30	0.49 ± 0.16 (6)	0.58 ± 0.15 (7)	0.67 ± 0.14 (8)
	40	2.61 ± 0.16 (6)	2.40 ± 0.15 (7)	2.49 ± 0.14 (8)
Hematocrit (%)[c]	24	10.5 ± 1.9 (4)	12.2 ± 1.7 (5)	8.3 ± 1.6 (6)
	30	16.3 ± 1.6 (6)	17.7 ± 1.4 (7)	15.4 ± 1.3 (8)
	40	30.9 ± 1.6 (6)	30.2 ± 1.4 (7)	26.3 ± 1.3 (8)
Hemoglobin[d] (g/100 ml)	24	1.58 ± 0.40 (4)	1.22 ± 0.36 (5)	1.39 ± 0.33 (6)
	30	2.89 ± 0.33 (3)	3.21 ± 0.30 (7)	2.85 ± 0.28 (8)
	40	7.04 ± 0.33 (6)	6.64 ± 0.30 (7)	5.88 ± 0.28 (8)
EPO (mU/ml)[e]	30	12.39 ± 0.90 (6)	13.0 ± 0.84 (7)	8.20 ± 0.78 (8)
	40	9.35 ± 0.90 (6)	10.8 ± 0.84 (7)	6.98 ± 0.78 (8)

[a] Least-squares means ± SEM; number of pregnant gilts in parentheses.
[b] Red blood cells increased ($p < 0.01$) with day of gestation.
[c] Percent hematocrit increased ($p < 0.01$) with day of gestation and was greater ($p < 0.01$) in WC than in MS.
[d] Hemoglobin concentrations increased ($p < 0.01$) with day of gestation.
[e] EPO (plasma erythropoietin) levels were greater ($p < 0.01$) on Day 30 than on Day 40 and were greater ($p < 0.01$) in WC than in MS.

TABLE 4. Hematological measures: maternal.[a]

Parameter	Day of gestation	WC INT	WC UHO	MS
RBC/ul ($\times 10^6$)[b]	24	9.41 ± 0.76 (5)	8.91 ± 0.69 (6)	9.64 ± 0.64 (7)
	30	9.11 ± 0.69 (6)	9.47 ± 0.64 (7)	7.78 ± 0.56 (8)
	40	11.57 ± 0.69 (6)	9.78 ± 0.64 (7)	8.14 ± 0.60 (8)
Hematocrit (%)[c]	24	45.4 ± 1.5 (5)	41.0 ± 1.4 (6)	41.9 ± 1.3 (7)
	30	47.2 ± 1.4 (6)	45.1 ± 1.3 (7)	40.1 ± 1.2 (8)
	40	43.3 ± 1.4 (6)	46.9 ± 1.3 (7)	40.5 ± 1.2 (8)
Hemoglobin[d] (g/100 ml)	24	15.6 ± 0.6 (5)	13.3 ± 0.5 (6)	12.6 ± 0.5 (7)
	30	12.7 ± 0.5 (6)	13.7 ± 0.5 (7)	11.9 ± 0.5 (8)
	40	13.2 ± 0.5 (6)	14.1 ± 0.5 (7)	12.0 ± 0.5 (8)
EPO (mU/ml)[e]	30	8.36 ± 0.76 (6)	8.16 ± 0.70 (7)	8.49 ± 0.66 (8)
	40	9.87 ± 0.76 (6)	11.88 ± 0.70 (7)	10.31 ± 0.66 (8)

[a] Least-squares means ± SEM; number of pregnant gilts in parentheses.
[b] Total RBC numbers were greater in INT than in UHO ($p < 0.05$) or MS ($p < 0.01$) on Day 40.
[c] Percent hematocrit was greater ($p < 0.05$) in INT than in UHO on Day 24 and was greater ($p < 0.01$) in WC than in MS on both Day 30 and Day 40.
[d] Hemoglobin concentrations were greater ($p < 0.01$) on Day 24 in INT vs. UHO or MS.
[e] EPO (plasma erythropoietin) levels were greater ($p < 0.01$) on Day 40 than on Day 30.

Two-Dimensional PAGE Analysis of Fetal Liver Protein Secretion

The ratio of TF to total counts increased with day of gestation ($p < 0.01$) and was higher ($p < 0.05$) in WC than in MS (Table 6). Protein 5 ratio was higher ($p < 0.01$) on Day 30 or Day 40 than on Day 24 (Table 6).

DISCUSSION

In this study we investigated the development of the fetal erythropoietic system in late embryonic and early fetal swine, and compared hematological measures and cultured liver protein secretion in fetuses from pigs of different breeds (WC and MS) and uterine environments (WC INT and UHO). Fetal weight, fetal liver weight, placental weight, fetal blood cell numbers, fetal hematocrit, and fetal hemoglobin concentrations all increased with day of gestation, as did the total percentage of circulating erythrocytes. Our data strongly indicate that the fetal erythropoietic system is functioning at this period of gestation in the pig and is maturing rapidly during a time period that is critical for survival in a crowded uterine environment [33].

Fetal weight data (Table 2) in this study agree with previous data [4] indicating that WC fetuses were heavier than MS fetuses on Day 30. No significant treatment effects were observed for placental weight (Table 2), although in another study WC pigs had higher placental weights than did MS [4]. This is most likely due to the smaller number of gilts used in the current study. Interestingly, covariate analysis revealed that MS had relatively larger placentas than WC when adjusted for fetal weight. Also, liver weights were smaller on Day 24 in MS than WC with placental weight as a covariate. The physiological significance of these observations is unknown at this time, but they suggest that by growing more slowly, MS pigs may have an advantage by having more placenta per gram of fetus than do WC pigs.

A previous study of fetal pig hematology indicated a Day 30 erythrocyte count of 560 000/μl and a hematocrit of 20.4% in Yorkshire and/or Duroc fetuses [43], which is similar to the values obtained in this study. However, the mean reticulocyte percentage was very low (1.10%) in that study [43] compared to this study (~20%; Table 5) on Day 30. Another earlier study [44] had values of 290 000 RBC/

TABLE 5. Differential cell counts of erythroid precursors in fetal blood.[a]

Cell type	Day of gestation	WC INT	WC UHO	MS
Basophilic erythroblast[b]	24	5.41 ± 0.81 (5)	3.88 ± 0.74 (6)	6.83 ± 0.69 (7)
	30	1.66 ± 0.64 (8)	0.88 ± 0.74 (6)	0.57 ± 0.69 (7)
	40	0.00 ± 0.74 (6)	0.07 ± 0.69 (7)	0.03 ± 0.64 (8)
Polychromatic erythroblast[c]	24	90.09 ± 3.65 (5)	86.54 ± 3.33 (6)	79.73 ± 3.08 (7)
	30	26.54 ± 2.88 (8)	26.25 ± 3.33 (6)	17.01 ± 3.08 (7)
	40	0.41 ± 3.33 (6)	0.36 ± 3.08 (7)	0.34 ± 2.88 (8)
Orthochromatic erythroblast[d]	24	4.84 ± 3.45 (5)	9.49 ± 3.15 (6)	13.28 ± 2.91 (7)
	30	42.9 ± 2.72 (8)	42.19 ± 3.15 (6)	49.17 ± 2.91 (7)
	40	5.11 ± 3.15 (6)	3.15 ± 2.91 (7)	4.72 ± 2.72 (8)
Reticulocyte[e]	30	26.06 ± 3.42 (8)	27.23 ± 3.95 (6)	27.27 ± 3.66 (7)
	40	28.62 ± 3.95 (6)	35.25 ± 3.66 (7)	19.96 ± 3.42 (8)
Erythrocyte[f]	24	0.02 ± 2.42 (5)	0.09 ± 2.21 (6)	0.15 ± 2.04 (6)
	30	2.84 ± 1.91 (8)	3.44 ± 2.21 (6)	5.98 ± 2.04 (7)
	40	67.41 ± 2.21 (6)	61.53 ± 2.04 (7)	74.70 ± 1.91 (8)

[a] Least-squares means ± SEM for percentage of erythroid precursor cell populations; number of pregnant gilts are in parentheses.
[b] The percentage of basophilic erythroblasts decreased ($p < 0.01$) with age.
[c] The percentage of polychromatic erythroblasts decreased ($p < 0.01$) with age.
[d] The percentage of orthochromatic erythroblasts was greater ($p < 0.01$) on Day 30 than on Day 24 or Day 40 and was greater ($p < 0.01$) in MS or UHO than in INT on Day 24.
[e] No significant effects of age or treatment were observed for reticulocyte percentage.
[f] The percentage of erythrocytes increased ($p < 0.01$) with age and was greater ($p < 0.01$) on Day 40 in MS than in WC.

TABLE 6. Two-dimensional (2-D) PAGE analysis of fetal liver explant cultures.[a]

Spot ID	Day of gestation	WC INT	WC UHO	MS
Transferrin ($\times 10^{-2}$)[b]	24	0.55 ± 0.17 (5)	0.62 ± 0.16 (6)	0.37 ± 0.17 (5)
	30	0.99 ± 0.16 (6)	1.02 ± 0.15 (7)	0.66 ± 0.16 (6)
	40	1.69 ± 0.17 (5)	1.18 ± 0.15 (7)	1.46 ± 0.14 (8)
Alpha-fetoprotein ($\times 10^{-2}$)[c]	24	4.33 ± 0.79 (5)	4.43 ± 0.72 (6)	2.09 ± 0.79 (5)
	30	4.02 ± 0.72 (6)	5.06 ± 0.67 (7)	4.72 ± 0.67 (6)
	40	4.96 ± 0.72 (6)	3.05 ± 0.66 (7)	3.56 ± 0.67 (7)
Retinol-binding protein ($\times 10^{-3}$)[d]	24	1.07 ± 0.22 (5)	1.25 ± 0.20 (6)	0.87 ± 0.22 (5)
	30	0.96 ± 0.20 (6)	1.25 ± 0.18 (7)	0.82 ± 0.18 (6)
	40	0.75 ± 0.20 (6)	0.65 ± 0.18 (7)	0.89 ± 0.17 (8)
Protein 4 ($\times 10^{-3}$)[e]	24	0.06 ± 0.52 (5)	0.11 ± 0.48 (6)	0.10 ± 0.52 (5)
	30	0.87 ± 0.48 (6)	0.46 ± 0.44 (7)	1.37 ± 0.44 (6)
	40	1.15 ± 0.48 (6)	1.50 ± 0.44 (7)	0.91 ± 0.41 (7)
Protein 5 ($\times 10^{-3}$)[f]	24	0.07 ± 1.14 (5)	0.09 ± 1.04 (6)	0.06 ± 1.14 (5)
	30	4.46 ± 1.04 (6)	1.76 ± 0.96 (7)	7.80 ± 0.96 (6)
	40	4.44 ± 1.04 (6)	5.91 ± 0.96 (7)	7.28 ± 0.90 (8)

[a] Least-squares means ± SEM; data are expressed as the ratio of radioactivity contained in individual protein spots punched from 2-D gels to the total amount of radioactivity contained in nondialyzable macromolecules added to the gel; number of pregnant gilts in parentheses.
[b] The ratio of transferrin increased ($p < 0.01$) with age and was greater ($p < 0.05$) in WC than in MS.
[c] No significant effects of day or treatment were observed for alpha-fetoprotein.
[d] No significant effects of day or treatment were observed for retinol-binding protein.
[e] No significant effects of day or treatment were observed for protein 4.
[f] Protein 5 ratio was greater ($p < 0.01$) on Day 30 or Day 40 than on Day 24.

μl on Day 26 and 390 000 RBC/μl on Day 32, which are similar to values in this study (Table 3), but reticulocyte percentages at those ages were much higher (99% and 100%, respectively) than observed on Day 30 in this study (Table 5). Other previous studies of pig hematology did not investigate these parameters during early gestation [45, 46]. The reason for these differences between studies is unknown, but they may be due to differences in methodologies.

Regression analysis revealed a significant regression on Day 40 between fetal weight and hematocrit percentage, and fetal weight and hemoglobin, which differed between intact uterine environments (WC INT and MS) and UHO. Examination of the data suggests that intrauterine crowding results in some fetuses with lower than average body weight and hematocrit percentage and/or blood hemoglobin. It is possible that fetuses in a normal intrauterine environment are not stressed as much (e.g., insufficient nutrients) as those in a crowded environment and therefore do not show this relationship. This observation supports the hypothesis that a crowded uterine environment may indeed affect growth and hematological parameters, which could ultimately result in fetal loss. It was hoped that all "crowded" fetuses might display some anomalies due to crowding. However, it is likely that only those fetuses that will eventually be lost show any differences from fetuses in an intact uterine environment.

EPO in the Swine Fetus

EPO, in conjunction with its receptor, is a growth factor for erythroid cells, particularly the late burst-forming units and colony-forming units that precede the erythroblast series [47], and thus tightly controls the number of circulating RBC. To detect EPO in porcine plasma, we used a heterologous RIA procedure with a polyclonal antibody generated against human EPO. Porcine EPO is not currently available, and thus levels of EPO in porcine plasma reported are indicative of relative levels and most likely underestimate absolute levels. EPO was detected in the plasma of fetal swine on Day 30 and Day 40 (Table 3). The relative decline in EPO between these ages may indicate a physiological difference in fetal erythropoiesis, perhaps due to the rapid expansion of the erythron (circulating RBC plus erythropoietic tissue [40]) between Day 30 and Day 40. The physiological significance of higher relative plasma EPO values in WC than in MS fetuses on Day 30 and Day 40 is not yet clear but is coincident with lower hematocrits on Day 40 in MS. Not only is EPO involved in production of RBC, but it also plays a role as a mitogen for murine fetal liver stromal cells in vitro [48] and thus may play a role in the growth of the liver at this time in the fetal pig. It is thought that EPO is produced by both the liver and the kidney in the adult animal, with the kidney producing the most significant amounts. The liver is thought to produce the majority of circulating EPO in the fetus [47]. However, EPO was not detected by RIA in cytosol preparations of fetal liver or immunohistochemically in fetuses in this study (data not shown). Thus, the source of circulating EPO in the swine fetus is unknown at this time. A previous study also could not detect EPO in liver extracts, despite EPO mRNA localization in the liver, indicating that EPO may be immediately secreted upon production [49] and thus be below detectable levels. A previous study in sheep and monkeys [50] showed that maternal EPO does not cross the placenta, so it is unlikely that circulating EPO originates from a maternal source. A recent study of human placenta indicated that immunoreactive EPO as well as EPO mRNA was present in the human trophoblast [51], raising the interesting possibility that the porcine placenta may produce EPO as well. Further study of the expression of EPO mRNA and protein in the pig will be required to identify the source of circulating fetal EPO.

Differential Cell Counts of Erythroid Precursors in Fetal Blood

The dramatic changes in erythroid precursor populations and the 10-fold increase in RBC concentrations seen in this study are compelling evidence of the critical nature of this time period (Day 24-Day 40) in the development of the erythron. The percentage of non-nucleated reticulocytes/erythrocytes changes from < 1.0% on Day 24 to roughly 90% of the total population of circulating RBC in just 17 days, while the percentage of PE drops from 80% to < 10% during the same time period (Table 5). These results

suggest the onset of production of a factor or factors that induce or promote maturation of the precursor cells during this time period. Significant differences (due to day of gestation) were observed for all cell types except reticulocytes. As expected, the more "primitive" precursors, BE and PE, were more prevalent on Day 24. OE were highest on Day 30, whereas erythrocytes were highest on Day 40. MS fetuses had a significantly lower percentage of PE, a more primitive precursor, than did WC, but a higher percentage of OE on Day 24, and a higher percentage of erythrocytes on Day 40 (Table 5). These data suggest that MS fetuses may produce more mature erythroid cells earlier in gestation than do WC fetuses. Further, WC fetuses had higher hematocrits than MS, despite no statistical difference in total circulating RBC between the two breeds. The smaller volume of the more mature erythroid precursors [40] in the MS fetus could contribute to this observation.

Two-Dimensional PAGE Analysis of Fetal Liver Protein Secretion

Data from the 2D-PAGE analysis indicate that the secretion of TF and protein 5 by the liver are both increasing at this critical time for fetal erythropoiesis (Table 6). Transferrin is an iron transport protein in the pig fetus and is important (along with uteroferrin) in supplying this mineral to developing erythropoietic and other tissues [30]. Total iron in the fetal liver increases between Day 30 and Day 45 in the pig, as does iron in allantoic fluid [31], where uteroferrin is metabolized and gives up iron to TF [30]. The significance of the overall higher ratio of TF in WC than in MS is unknown at this time. Protein 5 has yet to be identified, and further characterization will be required to ascertain its potential role in porcine fetal erythropoiesis. Alpha-fetoprotein was chosen for analysis in this study since it is a major secreted liver protein in the fetus during the time period studied [52] and appeared to have a constant level of expression, as was observed.

Conclusions

The present study provides data regarding the progression of erythropoiesis in late embryonic and early fetal swine of different breeds and uterine environments. These data indicate that the time period between Day 24 and Day 40 is indeed a critical time in the progression of erythropoiesis, since the circulating blood cell populations increase dramatically and change from primarily primitive precursors to almost entirely mature erythrocytes during this time. These data also indicate that the MS fetuses differ from WC fetuses in this process. Most parameters studied were generally lower in MS than in WC, except that MS fetuses had a somewhat more mature erythron. This study also indicates that the uterine environment had little overall effect on erythropoiesis in WC pigs but did affect fetal survival. The relationship of fetal weight and hematocrit/hemoglobin found for fetuses in the UHO group suggests that crowding may compromise erythropoiesis in small fetuses. However, all fetuses were not subjected to all the procedures in this study in order that a variety of procedures could be used to provide baseline information. Further studies focused on a few parameters that can be measured on every fetus are needed to fully evaluate whether abnormal erythropoiesis may be involved in fetal losses due to crowding.

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